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Remarkable advances in the treatment of urologic malignancies have recently been made. Monoclonal antibodies selective for a variety of normal and malignant urologic tissues have been useful in defining normal antigens and tumor-associated antigens and have potential as diagnostic and immunotherapeutic agents. In renal cancer, monoclonal antibodies can define serum markers, radiolabel tumor xenografts, and assist in specific tissue

[CANCER RESEARCH 53, 227–230, January 15, 1993]

Advances in Brief

M lecular Cl ning f a C mplementary DNA Encoding a Prostate-specific Membrane Antigen[1]

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Abstract

Recently, a novel M_r 100,000 prostate-specific membrane glycoprotein (PSM) has been detected by the prostate-specific monoclonal antibody 7E11-C5, raised against the human prostatic carcinoma cell line LNCaP. The PSM antigen is expressed exclusively by normal and neoplastic prostate cells and metastases. We now report the molecular cloning of a full-length 2.65-kilobase complementary DNA encoding the PSM antigen from a human LNCaP complementary DNA library by polymerase chain reaction using degenerate oligonucleotide primers. Analysis of the complementary DNA sequence has revealed that a portion of the coding region, from nucleotide 1250 to 1700, has 54% homology to the human transferrin receptor mRNA. The deduced polypeptide has a putative transmembrane domain enabling the delineation of intra- and extracellular portions of this antigen. In contrast to prostate-specific antigen and prostatic acid phosphatase which are secreted proteins, PSM as an integral membrane protein may prove to be effective as a target for imaging and cytotoxic targeting modalities.

Introduction

Prostate cancer represents the most common malignancy in American males and is the second leading cause of cancer-related death in the male population (1). The disease has diverse manifestations, from slow growing, indolent primary lesions to aggressive, refractory metastatic disease, with a predilection toward bone metastases. PAP[3] was one of the earliest serum markers for detecting metastatic spread of prostate cancer (1); this marker has been augmented in recent years by PSA (1). PSA has been shown to correlate with tumor burden, serve as an indicator of metastatic involvement, and provide an excellent parameter for following the response to surgery, irradiation, and androgen ablation therapy in patients with prostate cancer. Both of these proteins are secreted and are readily measured in the serum, as well as in prostatic secretions. The LNCaP human prostate cancer cell line was established from a metastatic lymph node from a heavily pretreated patient with hormone-refractory prostate carcinoma (2). This cell line serves as the best *in vitro* model for human prostatic carcinoma in that it possesses an aneuploid male karyotype, maintains prostatic differentiation functionality in that it produces PAP and PSA, and expresses a high affinity androgen receptor. Cell membranes were isolated from these cells and mice were immunized with them to form hybridomas. A prostate-specific monoclonal antibody was generated using spleen cells of mice immunized with LNCaP cell membranes and designated 7E11-C5 (3). The antibody staining exhibited a membrane location with LNCaP cells reacting strongly. Both benign and neoplastic prostate cells stained positively, with more intense staining seen with malignant cells. Lymph node and bone metastases also stain positively with the antibody, with the highest expression seen in hormone-refractory lesions (4). The epitope of the antibody has been shown to include a carbohydrate portion of the PSM antigen and the antigen has an apparent molecular weight of approximately 100,000 on SDS-polyacrylamide gel electrophoresis (5). In this paper, we report the molecular cloning of a full-length cDNA encoding the M_r 100,000 prostate-specific membrane antigen.

Materials and Methods

Cells and Reagents. The LNCaP, DU-145, and PC-3 cell lines used were obtained from the American Type Culture Collection. Details regarding the development of these cell lines and their characteristics have been published previously (2, 6, 7). Unless specified otherwise, LNCaP cells were grown in RPMI 1640 supplemented with L-glutamine, nonessential amino acids, and 5% fetal calf serum (Gibco-BRL) in a CO_2 incubator at 37°C. DU-145 and PC-3 cells were grown in minimal essential medium supplemented with 10% fetal calf serum. All media was obtained from the MSKCC Media Preparation Facility. Routine chemical reagents were obtained from Sigma Chemical Company, St. Louis, MO. The modified 7E11-C5 monoclonal antibody to the PSM antigen (CYT-356) was obtained from Cytogen Corporation, Princeton, NJ.

Immunoprecipitation of the PSM Antigen. LNCaP cells were starved in methionine-depleted RPMI for 2 h, after which [^{35}S]methionine was added at 100 μCi/ml and the cells were grown for another 16–18 h. Cells were then washed and lysed by addition of 1 ml of lysis buffer [1% Triton X-100, 50 mM Hepes (pH 7.5), 10% glycerol, 15 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, and 1 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid] and incubated for 20 min at 4°C. Lysates were precleared by mixing with Pansorbin cells (Calbiochem) for 90 min at 4°C. Cell lysates were then mixed with protein A-Sepharose CL-4B beads (Pharmacia) previously bound with CYT-356 monoclonal antibody and rabbit anti-mouse IgG (Accurate Scientific) for 4 h at 4°C. Beads were then washed with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, and 2 mM sodium *o*-vanadate buffer, resuspended in Laemmli sample loading buffer, and denatured prior to electrophoresing on a 10% SDS-PAGE gel at 10 mA overnight. Gels were dried down at 60°C in a vacuum dryer and autoradiographed for 16–24 h at −70°C. For the large scale purification of 5–10 μg of PSM antigen, the above procedure was repeated using approximately 6 × 10^7 LNCaP cells. The immunoprecipitation product was pooled and loaded into two lanes of a 10% SDS-PAGE gel and electrophoresed for 16 h at 10 mA. Proteins were electroblotted onto nitrocellulose membranes and stained with Ponceau red to visualize the proteins.

Peptide Microsequencing. This work was performed with the assistance of the Sloan-Kettering Institute Microchemistry Core Facility: Briefly, the M_r 100,000 PSM antigen band was excised from the membrane, solubilized, and digested proteolytically with trypsin. High performance liquid chromatography was performed on the digested sample using a HPLC Applied Biosystems Model 171C, and clear dominant peptide peaks were selected and sequenced on a modified post-liquid Applied Biosystems Model 477A Protein/Peptide Microsequencer (8). Nine peptides were sequenced ranging in size from 7 to 22 amino acids and all were screened for homology with the Genbank database and found to be unique. A similar technique was used to sequence the amino terminus of the PSM antigen and it was determined that it was in fact blocked, and no protein sequence was obtained.

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[1] The MSKCC Microchemistry Core Facility is supported in part by NIH Grant P30-CA-08748. R. S. I. receives partial support from NIH Training Grant CA-09501-07. Genback Accession Number M99487. PSM, Homo sapiens, 2653 base pairs.

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[3] The abbreviations used are: PAP, prostatic acid phosphatase; PSA, prostate-specific antigen; PSM, prostate-specific membrane glycoprotein; SDS, sodium dodecyl sulfate; cDNA, complementary DNA; MSKCC, Memorial Sloan-Kettering Cancer Center; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

RNA Isolation. Total cellular RNA was isolated from LNCaP cells by standard techniques (9). Polyadenylate-enriched RNA was prepared from total RNA by oligo-deoxythymidylate cellulose chromatography (10).

PCR with Degenerate Primers. Sense and antisense 5′-unphosphorylated degenerate oligonucleotide primers 17 to 20 nucleotides long corresponding to portions of the previously sequenced peptides were synthesized on an Applied Biosystems Model 394A DNA Synthesizer. These primers consisted of mixtures of 32 to 144 different sequences, in order to account for the degeneracy of the genetic code. PCR (11) was performed on a Perkin-Elmer Model 480 DNA Thermal Cycler, using a cDNA template prepared by reverse transcribing LNCaP mRNA with Superscript reverse transcriptase (Gibco-BRL) according to the manufacturer's recommendations. The PCR profile used was 94°C for 30 s, 45–55°C for 1 min (varied with the T_m of the primers used), followed by 72°C for 2 min. This was carried out for 30 cycles. Reactions were performed in a total volume of 50 µl containing 5 µl 10× PCR buffer (166 mM NH_4SO_4-670 mM Tris, pH 8.8-2 mg/ml bovine serum albumin), 5 µl 2.5 mM deoxynucleotide triphosphate mix, 5 µl Primer mix (0.5–1.0 µg each of sense and antisense primers), 5 µl 100 mM β-mercaptoethanol, 2 µl cDNA template, 5 µl 25 mM $MgCl_2$, 2 µl diluted Taq polymerase at 0.5 unit/µl (Promega), and 21 µl dH_2O.

Cloning of PCR Products. PCR products were cloned into the pCR II plasmid vector using the TA cloning system (Invitrogen). These plasmids were transformed into competent *Escherichia coli* cells using standard methods (12) and plasmid DNA was isolated using Magic Minipreps (Promega) and screened by restriction analysis.

DNA Sequencing of PCR Products. TA clones were then sequenced by the dideoxy method (13) using Sequenase (United States Biochemical). From 3 to 4 µg of each plasmid were denatured with NaOH and ethanol precipitated. Labeling reactions were carried out according to the manufacturer's recommendations using [^{35}S]dATP (NEN), and the reactions were terminated as discussed in the same protocol. Sequencing products were then analyzed on 6% polyacrylamide/7 M urea gels run at 120 W for 2 h. Gels were fixed for 20 min in 10% methanol/10% acetic acid, transferred to Whatman No. 3MM paper, and dried down in a vacuum dryer for 2 h at 80°C. Gels were then autoradiographed at room temperature for 16–24 h. Confirmation of correct clones was determined by reading DNA sequences adjacent to primer sequences looking for predicted peptide sequences that agreed with our peptide sequences.

cDNA Library Construction/Cloning of Full-Length cDNA. A cDNA library from LNCaP mRNA was constructed using the Superscript plasmid system (Gibco-BRL). The library was transformed using competent DH5-α cells (Gibco-BRL) and plated onto 100-mm plates containing L-Broth plus 100 µg/ml of carbenicillin. Plates were grown overnight at 37°C and colonies were transferred to nitrocellulose filters. Filters were processed and screened following techniques described by Grunstein and Hogness (14), using the 1.1-kilobase partial cDNA homologous probe, radiolabeled with [^{32}P]dCTP by random priming (15). Positive colonies were sequenced by the Sequenase method as described previously.

Northern Analysis of PSM Gene Expression. Analysis of PSM mRNA was performed according to previously described techniques (16). Ten µg of total RNA were denatured and electrophoresed through 1.1% agarose/formaldehyde gels at 60 mA for 8 h. RNA was then transferred to Nytran nylon membranes (Schleicher and Schuell) by pressure blotting in 10× standard saline-citrate with a Posi-blotter (Stratagene). RNA was cross-linked using a UV Stratalinker (Stratagene) and then baked in a vacuum oven for 2 h at 80°C. Blots were prehybridized at 65°C for 2 h and subsequently hybridized with denatured ^{32}P-labeled random-primed cDNA probe. Blots were washed twice in 1× saline-sodium phosphate-EDTA/0.5% SDS at 42°C and twice in 0.1× saline-sodium phosphate-EDTA/0.5% SDS at 50°C for 20 min each. Membranes were air-dried and autoradiographed for 12–36 h at −70°C with Kodak X-Omat film.

Results

Immunoprecipitation of the PSM Antigen. In agreement with previous results obtained by Western analysis using the CYT-356 monoclonal antibody (5), immunoprecipitation of the PSM antigen from metabolically labeled LNCaP cells yielded a single protein species with an apparent molecular weight of 100,000 on SDS-PAGE electrophoresis (Fig. 1).

PSM Antigen Peptide Sequencing. Approximately 10 µg of PSM antigen were purified as described in "Materials and Methods" and we obtained the following 9 peptide sequences:

1. SLYESWTK
2. SYPDGXNLPGGGVQR
3. FYDPMFK
4. IYNVIGTLK
5. FLYXXTQIPHLAGTEQNFQLAK
6. GVILYSDPADYFAPDGVK
7. AFIDPLGLPDRPFYR
8. YAGESFPGIYDALFDIESK
9. TILFASWDAEEFGXXGSTEWAE

Each of these 9 peptide sequences was found within the predicted amino acid sequence translated from the PSM antigen cDNA with only a few minor changes, presumably due to limitations of the protein sequencing technology. An attempt was also made to sequence the amino terminus of the PSM antigen but no sequence data could be obtained and it was concluded that the amino terminus of the protein is blocked.

Polymerase Chain Reaction. Degenerate primers designed from peptides 5 and 9 listed above were used in the polymerase chain reaction to amplify a 1.1-kilobase partial cDNA which was confirmed correct by DNA sequencing by the identification of the above peptide sequences contained within it. This cDNA sequence was screened on the Genbank computer database (Los Alamos, NM) and was found to be unique.

Cloning of the Full-Length PSM Antigen cDNA. Using the 1.1-kilobase partial PSM cDNA as a hybridization probe, 4 cDNAs encoding the PSM antigen were detected in the LNCaP cDNA library. The complete sequence of the longest cDNA; clone 55A (2.65 kilobases) and its deduced protein sequence are shown in Fig. 2. The entire 1.1-kilobase partial cDNA sequence is contained within the full-length PSM cDNA without changes. The open reading frame is 750 amino acids with a predicted protein molecular weight of 84,000, excluding carbohydrate. The presence of 5 in-frame stop codons between nucleotides −120 and −94 indicates that the ATG at nucleotide +1 is probably the actual initiator codon. Partial sequence analysis of the other 3 cDNAs indicated that they are identical to clone 55A, except the 5′ ends of these cDNAs terminate at different positions

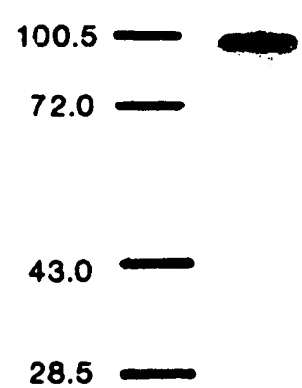

Fig. 1. Immunoprecipitation of the M_r 100,000 PSM antigen from [^{35}S]methionine-labeled LNCaP cells. Protein markers are shown on the *left*.

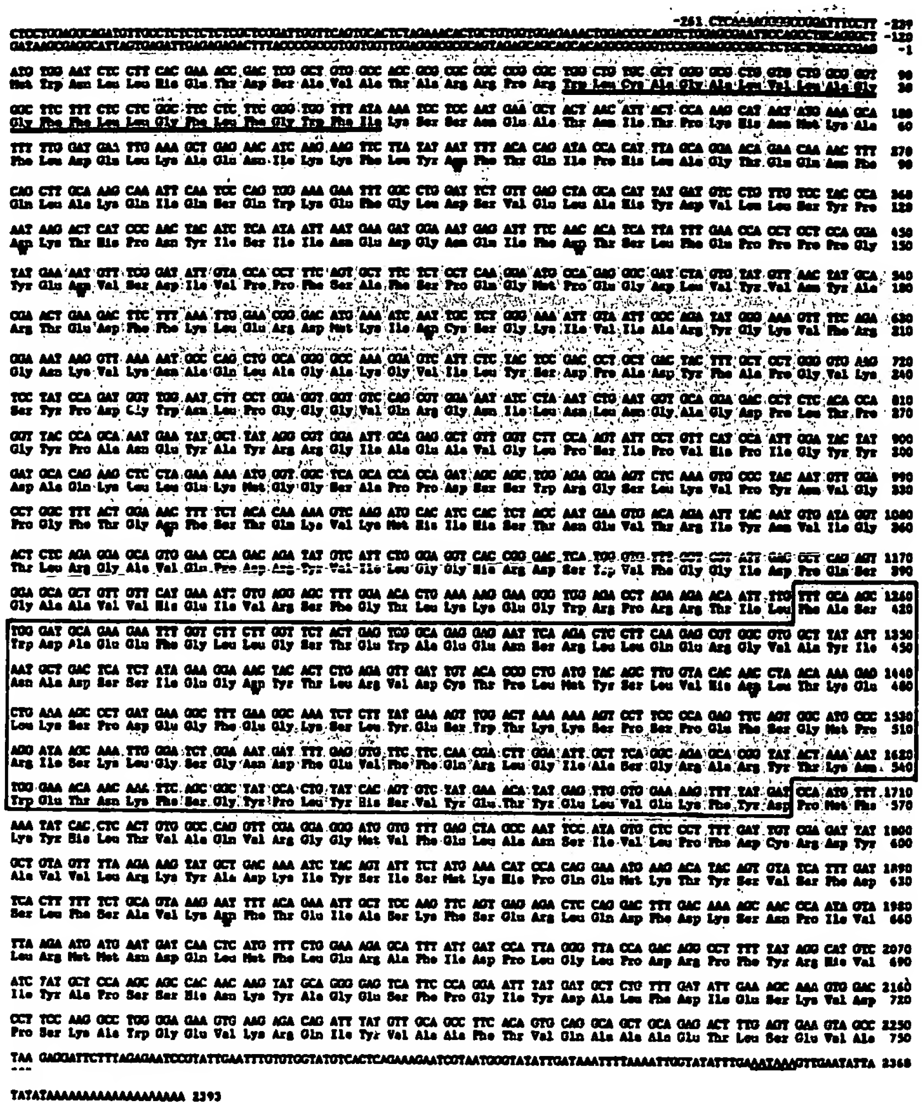

Fig. 2. Complete nucleotide sequence for the 2653-base pair PSM cDNA and the translated 750-amino acid predicted protein sequence. The transmembrane domain is *double-underlined* and polyadenylation signal is *single-underlined*. The area of homology with the human transferrin receptor is contained within the *boxed region*. Potential *N*-glycosylation sites are indicated with *asterisks*.

between the initiator codon and the 5′ end of clone 55A. The sequences of two of these additional cDNAs extend upstream of the five in-frame stop codons; thus these stop codons are probably part of authentic PSM RNA and not a 5′ artifact sequence. This provides further evidence that the coding region of PSM mRNA begins at nucleotide +1 of the sequence shown in Fig. 2.

Although the predicted protein sequence does not contain an NH_2-terminal hydrophobic signal sequence, a segment of hydrophobic residues from amino acids 20 through 43 forms a putative transmembrane domain (17). The major portion of the protein is COOH terminal to the transmembrane domain and contains multiple potential *N*-glycosylation sites. Greater than 54% homology on the nucleic acid level from nucleotides 1250 through 1700 within the coding region has been demonstrated with the human transferrin receptor mRNA by means of a Genbank homology search. Similar homologies have been identified for the chicken and rat transferrin receptor mRNAs. The entire full-length cDNA sequence has been compared to the Genbank database and confirmed to be unique.

Expressi n of the PSM Gene. Northern analysis using the PSM cDNA probe has revealed expression of a 2.8-kilobase message in the LNCaP cell line, with no expression in the DU-145 and PC-3 cell lines (Fig. 3). Expression of the PSM antigen appears to be limited to prostatic tissues, both benign and neoplastic, with no detectable expression in any of the nonprostatic tissues and cell lines tested to date.[4]

Discussion

Organ-specific antigens permit insight into the processes that occur uniquely within a particular tissue. The prostate is a very unusual organ in that with aging most organs atrophy, whereas the prostate gland almost invariably hypertrophies and in a very high percentage of cases develops a malignancy. A cell model that has proven valuable in fostering our understanding of prostatic cancer has been the LNCaP prostate cancer cell line. These cells express markers characteristic of prostatic epithelial cells such as PSA and PAP as well as a functi nal androgen receptor. These cells were used to immunize mice and resulted in the generation of the 7E11-C5 monoclonal antibody and its subsequent modified derivative, CYT-356, which we used to clone the PSM cDNA. The PSM antigen appears to have many interesting and potentially significant properties. The presence of 3 arginine residues at the NH_2-terminal end of the putative transmembrane domain suggests that the PSM protein is a type II integral membrane protein, with

[4] Unpublished data.

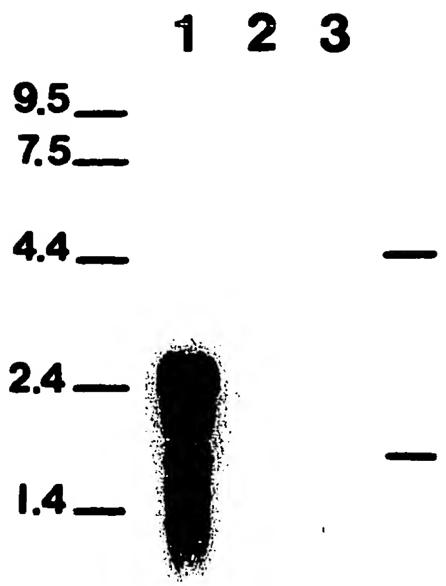

Fig. 3. Autoradiogram of Northern analysis revealing expression of 2.8-kilobase PSM message unique to the LNCaP cell line (*Lane 1*) and absent from the DU-145 (*Lane 2*) and PC-3 cell lines (*Lane 3*). RNA size ladder is shown on the *left* (kilobases), and 28S and 18S rRNA bands are indicated on the *right*.

a short NH_2-terminal region on the cytoplasmic side of the membrane and a large COOH-terminal domain on the extracellular side (17). This prediction is supported by the finding that removal of basic residues from the NH_2-terminal side of type II integral membrane protein transmembrane domains can reverse the orientation of such proteins in the membrane (18).

As an integral membrane protein unique to prostatic epithelial cells, the antigen or perhaps a specific PSM ligand may serve as an excellent site for use in the imaging and/or targeting of metastatic deposits. Indeed, current studies suggest that the CYT-356 antibody may be useful in imaging extraprostatic deposits of cancer cells (5). The CYT-356 antibody recognizes an epitope that is at least in part carbohydrate. It is possible that a unique peptide-recognizing antibody may have less nonspecific binding and that, additionally, multiple antibodies recognizing multiple areas of the PSM antigen may enhance the ability to image and treat metastatic prostate cancer.

PSA expression tends to decrease in hormone-refractory disease and bone metastases, while the expression of PSM appears to increase, again implying that it may provide an attractive target for therapy and diagnosis.

The homology to the human transferrin receptor is an interesting finding. It is of interest that the expressed prostatic secretions of patients with prostate cancer are enriched with respect to their content of transferrin and that prostatic cancer cells are rich in transferrin receptors (19). It was previously hypothesized that the microenvironment f bone w uld serve t stimulate prostatic cancer cell growth. This was recently observed to be the case, inasmuch as bone stroma cell transferrin dramatically stimulated the growth of metastatic prostatic cancer cell lines (20). In these experiments, the androgen receptor-negative DU-145 and PC-3 cell lines were used and LNCaP cells were not examined. Whether the PSM antigen interacts with transferrin or an ther ligand and possibly facilitates metastatic spread is presently being addressed in our laboratory. Transferrin may prove t be more than a transport m lecule, because apotransferrin has been sh wn to be mitogenic t some tumor cells (21).

Finally, we are presently developing new antibodies directed against peptide epitopes f the PSM antigen which are predicted to be highly antigenic, with the expectati n that these may be used to develop serum enzyme-linked immunosorbent assays, aid in tissue diagnoses, and serve as new agents for the immunotherapy of advanced, hormone-refractory prostate cancer.

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